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Award Number: W81XWH-06-1-0006

TITLE: Mechanism of Selenium Chemoprevention and Therapy in Prostate Cancer

PRINCIPAL INVESTIGATOR: Allen C. Gao, M.D., Ph.D.

CONTRACTING ORGANIZATION: Roswell Park Cancer Institute
                                            Buffalo, NY 14263

REPORT DATE: November 2007

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
         Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
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REPORT DOCUMENTATION PAGE

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1. REPORT DATE *(DD-MM-YYYY)* 01-11-2007	2. REPORT TYPE Annual	3. DATES COVERED *(From - To)* 15 OCT 2006 - 14 OCT 2007

4. TITLE AND SUBTITLE Mechanism of Selenium Chemoprevention and Therapy in Prostate Cancer	5a. CONTRACT NUMBER
	5b. GRANT NUMBER W81XWH-06-1-0006
	5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S) Allen C. Gao, M.D., Ph.D. E-Mail: gaoac@yahoo.com	5d. PROJECT NUMBER
	5e. TASK NUMBER
	5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Roswell Park Cancer Institute Buffalo, NY 14263	8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012	10. SPONSOR/MONITOR'S ACRONYM(S)
	11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT
Prevention trials demonstrated that selenium is a promising chemopreventive agent for prostate cancer. Selenium inhibited human prostate cancer cell growth, blocked cell cycle progression, and induced apoptotic cell death. We have demonstrated a novel mechanism of selenium anticancer action in which selenium markedly reduces androgen receptor (AR) expression and AR-mediated gene expression including prostate-specific antigen (PSA) in human prostate cancer cells in vitro and in vivo. Based on our novel finding that selenium disrupts AR signaling by reducing AR expression, it is conceivable that selenium (reducing AR expression) might improve the efficacy of androgen deprivation therapy. In this application, we will test the effects of selenium on prostate cancer therapy.

15. SUBJECT TERMS
Selenium, therapy, prostate cancer

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON USAMRMC
a. REPORT U	b. ABSTRACT U	c. THIS PAGE U	UU	21	19b. TELEPHONE NUMBER *(include area code)*

Standard Form 298 (Rev. 8-98)
Prescribed by ANSI Std. Z39.18

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Introduction

The goal of this application is to elucidate the importance of down regulation of AR signaling by multiple selenium compounds and select the best leading selenium compound for prostate cancer chemoprevention and therapy. In this application, we will further study the mechanisms of AR downregulation by multiple selenium compounds and functional significance of this down regulation in prostate cancer chemoprevention and therapy. Prevention trials demonstrated that selenium reduced prostate cancer incidence by 50%, establishing selenium as a promising chemopreventive agent for prostate cancer. Selenium inhibited human prostate cancer cell growth, blocked cell cycle progression at multiple transition points, and induced apoptotic cell death. We have demonstrated a novel mechanism of selenium anticancer action in which selenium markedly reduces androgen receptor (AR) expression and AR-mediated gene expression including prostate-specific antigen (PSA) in human prostate cancer cells in vitro and in vivo. Androgen signaling through androgen receptor (AR) plays an important role not only in maintaining the function of the prostate, but also in promoting the development of androgen-independent prostate cancer. AR signaling is often hyperactive in androgen-independent prostate cancer. A common treatment for prostate cancer is androgen deprivation. Although most men respond to androgen deprivation therapy initially, almost all relapse due to the growth of androgen-independent cancer cells. Most of the androgen deprivation treatments are either blocking androgen-AR binding or reducing the levels of androgen. Based on our novel finding that selenium disrupts AR signaling by reducing AR expression, a completely different mechanism from the current androgen deprivation therapy, it is conceivable that targeting AR signaling by a combination of androgen-deprivation therapy and selenium (reducing AR expression) might improve the efficacy of current androgen deprivation therapy. This concept was validated in vitro in which the combination of selenium and anti-androgen (Casodex) synergistically inhibited clonogenic ability of human prostate cancer cells, providing a rationale for in vivo validation of the combination of selenium and anti-androgen therapy for prostate cancer. The hypothesis is that anticancer effects of multiple selenium compounds are mediated, in part, by inhibition of AR activity and that decreased AR signaling may reduce the incident of prostate cancer and prevent or delay relapses after androgen deprivation therapy. The goal of this application is to elucidate the importance of down regulation of AR signaling by multiple selenium compounds and determine the best leading selenium compound for prostate cancer chemoprevention and therapy.

Body

Since the approval of this application, we have made significant progress of task 1 (i.e., To compare the effect of multiple selenium compounds and determine the molecular basis of the effects of multiple selenium compounds on AR expression (Months 1-8)*.*

MSA decreases AR mRNA stability Our results suggest that while MSA decreased AR mRNA levels at the transcriptional level, AR mRNA expression can also be regulated at post-transcriptional level. To examine whether MSA affects AR mRNA

stability, LNCaP cells that express functional AR were treated with or without 5 µM of MSA in the presence of actinomycin D (5 µg/ml) to stop de novo mRNA synthesis. The total RNA was isolated at different time points and AR mRNA levels were measured by Northern blot analysis. The half-life of AR mRNA was determined by comparison of mRNA levels over time between cells treated with or without actinomycin D, either in the presence or absence of MSA. Since actinomycin D is capable of inducing cell death, we monitored cell growth for a period of 24 h and did not observe cell death or growth inhibition with the concentration of actinomycin D used (5 µg/ml). We did not observe significant cell death or growth inhibition at 5 µM MSA over a period of 24 h in LNCaP cells. MSA treatment initially enhanced AR mRNA levels within 6 h. However, AR mRNA levels were significantly decreased by MSA compared to the control at 8 h. Figure 1 shows on the semi-log plot, the mean values of percentage of AR mRNA levels over time relative to respective time zero AR mRNA value as 100%. In MSA treated cells, AR half-life was reduced to about 7 h from 12 h in the control cells, suggesting that AR mRNA degradation was greatly accelerated in the presence of MSA after 6 h.
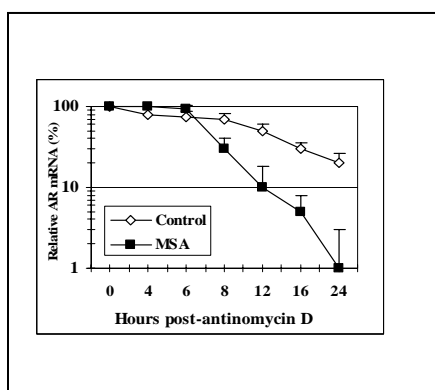

Figure 1 Effect of MSA on AR mRNA stability in LNCaP cells. The mRNA synthesis inhibitor antinomycin D (5 µg/ml) was added with or without 5 µM MSA at time 0. At specific time points, cells were harvested and total RNA as isolated by Northern blots. Points, means of three independent experiments plotted on semi-log scale relative to respective time zero AR mRNA value as 100%; bar, SD.

MSA increases AR protein turnover   We have demonstrated that MSA decreased the levels of AR mRNA and protein in LNCaP cells. We next examined the effect of MSA on AR protein degradation after new protein synthesis was blocked by cycloheximide as a potential mechanism for downregulation of AR protein level. The protein synthesis inhibitor cycloheximide (50 µg/ml) was added with or without 5 µM MSA at time 0. At specified time points, cells were harvested and the levels of AR protein were measured by Western blot using anti-AR antibody. In MSA-treated cells, the half-life of AR protein was reduced to 6 h from 21 h in the control cells (Fig. 2A), suggesting that AR protein degradation was greatly enhanced in the presence of MSA. Systematic protein degradation by the ubiquitin-proteasome system plays an important role in the maintenance of protein stability. Protein ubiquitination provides the recognition signal for the 26S proteasome, leading to protein degradation. Studies demonstrated that AR protein level in cells is regulated by systemic protein degradation pathways. To examine whether selenium induced AR protein degradation via ubiquitin-proteasome system, the 26S proteasome inhibitor MG132 was added to the cells treated with MSA. MG132 was able to retard MSA effect on AR protein levels (Fig. 2B), suggesting that MSA induced AR degradation via a proteasome-dependent pathway.
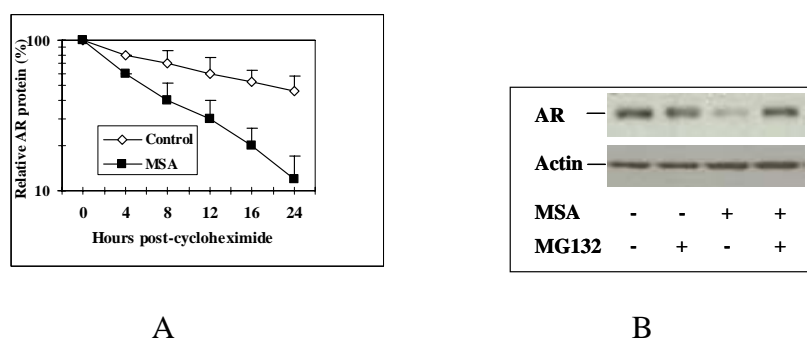
A   B

Figure 2. A. Effect of MSA on AR protein turnover in LNCaP cells. The protein synthesis inhibitor cycloheximide (50 µg/ml) was added with or without 5 µM MSA at time 0. At specific time points, cells were harvested and cell lysates were prepared. AR protein levels were determined by Western blot analysis using antibody specifically against AR and normalized to α-actin control. Points, means of three independent experiments plotted on semi-log scale relative to respective time zero AR value as 100%; bars, SD. **B.** Effect of MG132 on MSA induced AR protein degradation. MG132 (5 µM) was added to LNCaP cells together with cycloheximide (50 µg/ml) in the presence and absence of 5 µM of MSA. The cell lysates were prepared at 24 h. AR protein levels were determined by Western blot analysis using antibodies specifically against AR and α-actin as a control.

Selenium inhibits AR nuclear translocation AR typically translocates to the nucleus to exert its function on gene expression. To examine whether selenium affects the translocation of AR, Western blot analysis was performed using cell extracts from either cytosolic or nuclear extracts. LNCaP cells were cultured in charcoal stripped FBS for 3 days before adding 10 nM of DHT in the absence or presence of 10 µM MSA for 2 h. Nuclear and cytosolic fractions were prepared and used for Western blot analysis using the anti-AR antibody. DHT treatment increased the levels of AR protein expression in the nucleus which were reduced by the treatment with MSA (Fig. 3). In contrast, MSA had little effect on AR protein expression in the cytosol. The expression of RNA polymerase II (Pol II) and Hsp90 were used as markers for the integrity of the nuclear and cytosolic fractions, respectively. These results suggest that MSA suppresses AR signaling in part via interruption of AR nuclear translocation.
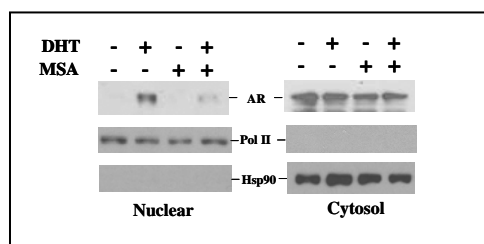

Figure 3. The effect of MSA on AR nuclear translocation. LNCaP cells were cultured in charcoal stripped FBS for 3 days and treated with 10 nM DHT with or without 10 µM MSA for 2 h. The cells were harvested for preparation of cytosolic and nuclear fractions and analyzed by Western blotting using antibodies against AR, Pol II, or Hsp90. The expression of Pol II and Hsp90 were used as markers for the integrity of the nuclear and cytosolic fractions, respectively.

Selenium inhibits the recruitment of coactivators and enhances the recruitment of corepressors to AR target genes AR interacts with coregulators to achieve maximal transactivation activity. To examine the effects of selenium on the recruitment of coregulators to the promoters of AR target genes, chromatin immunoprecipitation (ChIP) analysis was performed. DHT increased the recruitment of AR and TIF-2, SRC-1 to the promoter of PSA gene in the absence of MSA and this recruitment was greatly diminished in the presence of 5 µM MSA (Fig. 4). On the other hand, MSA treatment prevented the nuclear translocation of AR in the presence of hormone, thus the corepressors including SMRT and NCoR remain bound to the promoter of the PSA gene (Fig. 4). These results suggest that MSA-mediated reduction of AR activation may be due, at least in part, to a decrease in the recruitment of AR and its coactivators to the promoter of the AR target gene PSA, while maintains corepressors bound to the promoter.
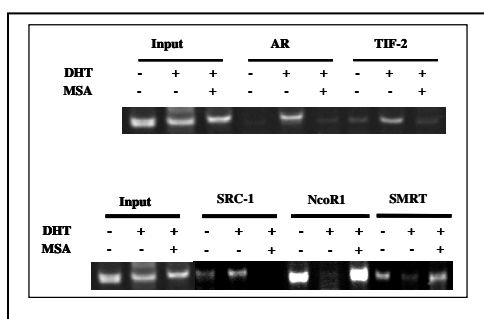

Figure 4. Effect of MSA on the recruitment of AR and coregulators to the promoter of an endogenous AR target gene, PSA. The *in vivo* binding of AR and coregulators to the PSA promoter was examined by the ChIP assay. LNCaP cells were cultured in charcoal stripped condition for 3 days. Soluble chromatin was prepared from cells treated with 10 nM DHT for 4 h (+) or untreated (-) in the presence (+) or absence (-) of 10 µM MSA and immunoprecipitated with antibodies against AR, TIF-2, SRC-1, SMRT, and NCoRI. Co-precipitated DNA was amplified by PCR using primers that flank the ARE in the PSA promoter region. The presence of total PSA promoter DNA in the soluble chromatin prior to immunoprecipitation was included as input.

We have made progress on task 3 during the 2nd year funding period (*To evaluate combination of selenium and anti-androgen therapies in mouse models of prostate carcinogenesis*)**.**

Combination of selenium and anti-androgen synergistically reduces AR transactivation
A common treatment modality for prostate cancer is androgen deprivation. The goal of these androgen deprivation treatments is either blocking androgen-AR binding or reducing the levels of androgen. Although anti-androgen treatment is effective, the anti-tumor effects can be temporary. Numerous studies have demonstrated that AR is expressed and AR signaling remains intact and is often hyperactive in androgen-independent prostate cancer. Selenium decreases AR expression and reduces AR activation provides a molecular basis for selenium chemoprevention and chemotherapy targeting AR signaling in prostate cancer. We hypothesize that an intervention strategy aimed at both blocking ligand binding (chemical or surgical

castration) and dampening AR expression (selenium treatment) would be achieve better therapeutic effect than either alone. To test this hypothesis, we first examined whether combination of selenium with anti-androgen, flutamide (Flu), can synergistically inhibit AR transactivation in human prostate cancer cells *in vitro*. C4-2 cells were transfected with ARE-containing luciferase reporter and treated with either flutamide or MSA alone, or the combination of flutamide and MSA. The ARE luciferase activity was reduced by either flutamide or MSA alone, however, combination of flutamide and MSA achieved much greater inhibition of ARE luciferase activity than either flutamide or MSA alone (**Fig. 5**), suggesting that blocking AR ligand binding and reducing AR expression may synergistically inhibit AR transactivation in prostate cancer cells.
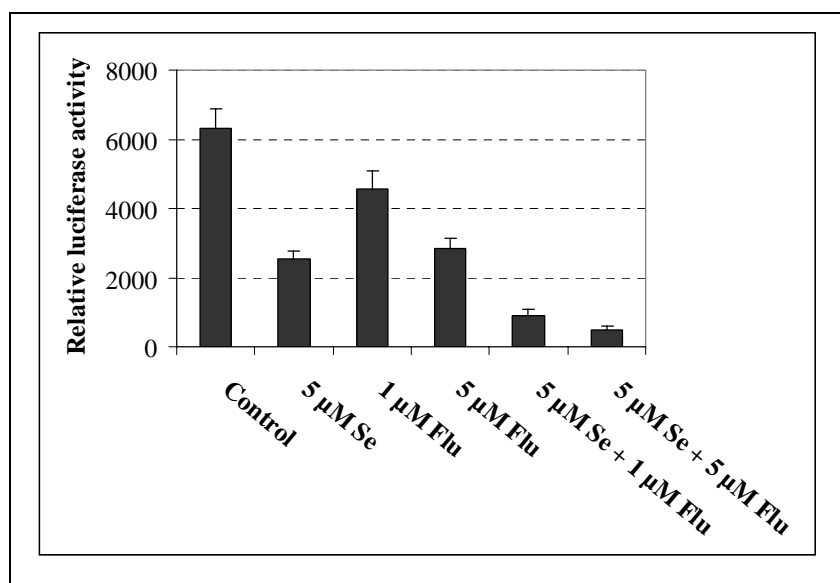

Fig. 5. The effect of combination of selenium (Se) and flutamide (Flu) on AR activation in C4-2 cells. C4-2 cells were transfected with plasmid containing androgen responsive element (ARE)-luc and treated with either Se or Flu alone or together as indicated. Luciferase activity was determined and normalized to the amount of protein.

The effects of combination of MSA and antiandrogen agents on clonogenic ability *in vitro* in C4-2 cells To test whether reduction of AR transactivation by the combination of flutamide with MSA affects cell clonogenic ability *in vitro*, C4-2 cells were treated with either flutamide or MSA alone or together, and clonogenic ability was determined. Treatment with flutamide or MSA alone reduced C4-2 clonogenic ability, combination of flutamide and MSA inhibited C4-2 clonogenic ability much greater than either one alone (**Fig. 6**), suggesting that combination of flutamide with MSA can synergistically inhibit C4-2 cell clonogenic ability *in vitro*.
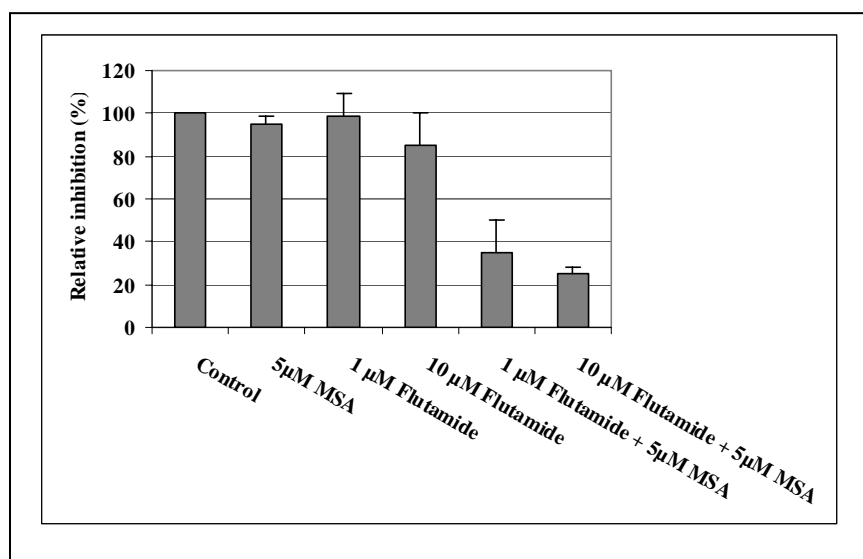
Fig. 6. The effects of combination of selenium (Se) and flutamide (Flu) treatment on clonogenic ability of C4-2 cells.

Effect of combination of selenium and anti-androgen on xenograft models of human prostate cancer

Having demonstrated that combination of selenium and anti-androgen agents can achieve better efficacy on inhibiting AR activation and cell clonogenic ability, we next test the effects of the combination on in vivo tumor growth. The effect of combination of methylselenocysteine (MSC) and hydroxyflutamide on tumor growth of C4-2 prostate cancer growth was demonstrated *in vivo*. The male nude mice were co-inoculated with 3×10^6 cells and Matrigel. When the tumors became palpable, the mice were divided into 4 groups with 8 mice in each group. One group was treated with 100 µg MSC, the rest groups were treated with 500 mcg of hydroxyflutamide, 500 mcg hydroxyflutamide plus 100 µg MSC, and vehicle control by i.p. injection daily for 12 days, respectively. Tumor volume was measured 3 times a week. As shown in **Fig. 7**, MSC + flutamide had a greater effect on suppression of tumor growth than flutamide alone.
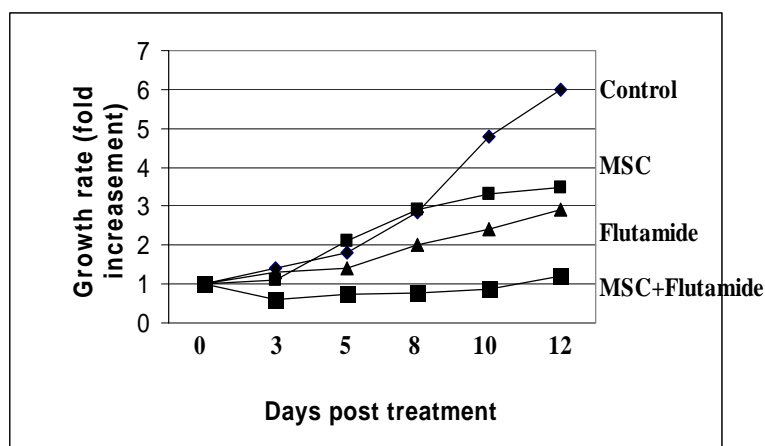
Figure 7. The effect of combination of MSC and hydroxyflutamide on tumor growth of C4-2 prostate cancer growth *in vivo*. The male nude mice were co-inoculated with 3×10^6 cells and Matrigel. When the tumor became palpable, the mice were divided into 4 groups with 8 mice in each group. One group was treated with 100 mcg MSC, the rest groups were treated with 500 mcg of hydroxyflutamide, 500 mcg hydroxyflutamide plus 100 mcg MSC, and vehicle control by i.p. injection daily for 12 days, respectively. Tumor volume was measured 3 times a week.

Effect of combination of MSC and flutamide treatment on AR, Akt expression

We previously demonstrated that selenium significantly suppressed AR expression and AR-regulated gene PSA expression in LNCaP cells *in vitro*. To determine whether selenium affects AR expression *in vivo*, we determined AR protein expression in the C4-2 bearing tumor tissues using Western blot. The levels of AR protein expression were considerable decreased by the treatment with either MSC or flutamide alone and by the combination of MSC and flutamide (**Fig. 8**). The expression of phosphorylate Akt was also decreased by the treatment with either MSC or flutamide alone, and the expression was further decreased by the combination of MSC and flutamide (**Fig. 8**).
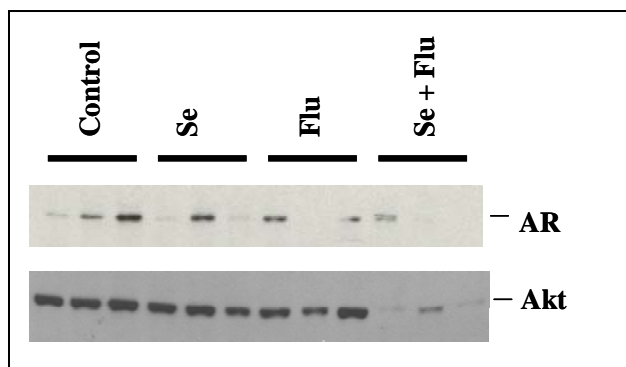

Fig. 8. AR and Akt protein expression in tumors by Western blot analysis. Nuclear extracts were isolated from tumors of individual mice and indicated and subjected to Western blot analysis.

Key research accomplishments

- We demonstrated that selenium downregulates AR signaling in prostate cancer cells.
- MSA decreases AR mRNA stability.
- MSA increases AR protein turnover.
- Selenium inhibits AR nuclear translocation.
- Selenium inhibits the recruitment of coactivators and enhances the recruitment of corepressors to AR target genes.
- Combination of selenium with antiandrogen significantly reduced the number of colony formation than either selenium or antiandrogen alone.
- Combination of selenium with antiandrogen has a greater effect on suppression of tumor growth than either flutamide or selenium alone.
- Combination of selenium with antiandrogen significantly reduced the levels of AR and Akt protein expression in tumors.

Reportable outcome

Publications:

Chun JY, Nadiminty N, Lee SO, Onate SA, Lou W, and Gao AC. Mechanisms of selenium down regulation of androgen receptor signaling in prostate cancer. *Molecular Cancer Therapeutics*, 5 (4): 913-918, 2006.

Abstract:

1. Lee SO, Chun JY, Nadiminty, N, and Gao AC. Selenium inhibits growth of LNCaP human prostate tumor accompanied by a decrease in the expression of androgen receptor and prostate-specific antigen (PSA). Innovative Minds in Prostate Cancer Today (IMPaCT) meeting. Proceedings P18-14, p206, Atlanta, GA, September 5-8, 2007.

Conclusions

- We demonstrated that selenium downregulates AR signaling via multiple pathways including decreases AR mRNA and protein expression, decreases AR mRNA stability, increases AR protein turnover, inhibits AR nuclear translocation, and affects the recruitment of coregulators to the androgen responsive genes.

- Combination of selenium and anti-androgen therapies has better antitumor effect than either selenium or anti-androgen alone.

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2. Dong Y, Lee SO, Zhang H, Marshall J, Gao AC and Ip C. Prostate specific antigen (PSA) expression is down-regulated by selenium through disruption of androgen receptor signaling. *Cancer Res*. 64 (1): 19-22, 2004.
3. Chun JY, Nadiminty N, Lee SO, Onate SA, Lou W, and Gao AC. Mechanisms of selenium down regulation of androgen receptor signaling in prostate cancer. *Molecular Cancer Therapeutics*, 5 (4): 913-918, 2006.
4. Lee SO, Chun JY, Nadiminty N, Trump DL, Ip C, Dong Y, and Gao AC. Monomethylated selenium inhibits growth of LNCaP human prostate cancer xenograft accompanied by a decrease in the expression of androgen receptor and prostate-specific antigen (PSA). *Prostate 66: 1070-1075,* 2006.

Appendix

1. Chun JY, Nadiminty N, Lee SO, Onate SA, Lou W, Gao AC. Mechanisms of selenium down-regulation of androgen receptor signaling in prostate cancer. Mol Cancer Ther 2006; 5 (4): 913-918.

2. Lee SO, Chun JY, Nadiminty, N, and Gao AC. Selenium inhibits growth of LNCaP human prostate tumor accompanied by a decrease in the expression of androgen receptor and prostate-specific antigen (PSA). Innovative Minds in Prostate Cancer Today (IMPaCT) meeting. Proceedings P18-14, p206, Atlanta, GA, September 5-8, 2007.

Mechanisms of selenium down-regulation of androgen receptor signaling in prostate cancer

Jae Yeon Chun, Nagalakshmi Nadiminty,
Soo Ok Lee, Sergio A. Onate, Wei Lou,
and Allen C. Gao

Departments of Medicine and Pharmacology and Therapeutics,
Roswell Park Cancer Institute, Buffalo, New York

Abstract

Prevention trials showed that selenium reduced prostate cancer incidence by 50%, establishing selenium as a promising chemopreventive agent for prostate cancer. Selenium inhibited human prostate cancer cell growth, blocked cell cycle progression at multiple transition points, and induced apoptotic cell death. Previous studies showed a novel mechanism of selenium anticancer action in which selenium markedly reduces androgen signaling and androgen receptor (AR) – mediated gene expression, including prostate-specific antigen (PSA), in human prostate cancer cells. The molecular mechanisms of selenium-mediated down-regulation of AR signaling are not clear. In this study, a systemic approach was taken to examine the modification of androgen signaling by selenium in human prostate cancer cells. In addition to reduced AR mRNA expression, selenium was found to initially increase the stability of AR mRNA within 6 hours while decreasing the stability of AR mRNA after 8 hours. Selenium increased AR protein degradation and reduced AR nuclear localization. Scatchard analysis indicated that selenium did not affect ligand binding to AR in LNCaP cells. Chromatin immunoprecipitation analyses showed that DHT increased the recruitment of AR and coactivators, such as SRC-1 and TIF-2, to the promoter of the *PSA* gene, and that recruitment was greatly diminished in the presence of 5 μmol/L selenium. On the other hand, selenium enhanced the recruitment of corepressors, such as SMRT, to the promoter of the *PSA* gene. Taken

together, these results suggest that selenium disrupts AR signaling at multiple stages, including AR mRNA expression, mRNA stability, protein degradation, nuclear translocation, and recruitment of coregulators. [Mol Cancer Ther 2006;5(4):913 – 8]

Introduction

The growth of prostate epithelial cells requires physiologic levels of androgen, both to stimulate proliferation and inhibit apoptotic death (1). Androgen binds to the androgen receptor (AR), which causes AR to bind to androgen-responsive elements in the promoters of androgen-regulated genes. This interaction is affected by many other transcription coregulators. These complex interactions among AR, androgen-responsive elements, and coregulators facilitate the activation or repression of genes regulating development, differentiation, and proliferation of target cells. Several androgen-responsive genes have been identified, including prostate-specific antigen (*PSA*) and *human glandular kallikrein 2* (2).

Selenium is an essential nutrient that has a chemopreventive effect against a variety of malignancies, including prostate cancer. A number of case-controlled epidemiologic studies have shown an inverse relationship between selenium status and prostate cancer risk (3–7). One of the most important studies of selenium as a chemopreventive agent is the Nutritional Prevention of Cancer study initiated by Clark et al. (8). Supplementation of people with selenized yeast was capable of reducing the overall cancer morbidity by nearly 50% (8). Although selenium treatment did not significantly affect the incidence of nonmelanoma skin cancers, patients receiving the supplement showed a significantly lower prevalence of developing lung (relative risk, 0.54), colon (relative risk, 0.42), or prostate cancer (relative risk, 0.37). Further analysis (9) reaffirmed the significant reduction in prostate cancer incidence by selenium (relative risk, 0.48; 95% confidence interval, = 0.28–0.80). The promising epidemiologic and prevention studies on selenium in prostate cancer provide the basis for the current Selenium and Vitamin E Chemoprevention Trial (10).

The biological activity of selenium is dependent on its chemical form. In general, inorganic selenium compounds, such as selenate or selenite, are known to produce genotoxic effects. Organic selenium-containing compounds, such as selenomethionine and methylselenocysteine, are better tolerated and exhibit anticarcinogenic activity. Methylseleninic acid (CH_3SeO_2H) was developed specifically for *in vitro* studies (11) because cultured cells respond poorly to selenomethionine (a commonly used selenium reagent) due to very low levels of β-lyase activity,

which is required for conversion of selenomethionine to the active methylselenol (12). The effect of physiologic concentrations of methylseleninic acid on cultured cells has been documented in several studies (11, 13–15).

Cell culture studies showed that selenium inhibited the growth of prostate cancer cell lines, including androgen-sensitive LNCaP and androgen-insensitive DU145 and PC3 cells (14–17). *In vivo* studies also support the antitumorigenic role of selenium in prostate cancer. Dietary supplementation of selenium resulted in reduction of tumor growth in PC3 tumors in mice (18). There are a number of potential mechanisms proposed for the antiproliferative effects of selenium, including antioxidant effects, enhancement of immune function, stimulation of apoptosis, and induction of cell cycle arrest (16). We recently showed that methylseleninic acid is able to decrease markedly AR transcript and protein levels (14). The expression of *PSA*, a well-known androgen-regulated gene, is also inhibited by methylseleninic acid (13, 14).

The down-regulation of AR signaling by selenium provides an important mechanism for selenium prostate cancer chemoprevention. However, the molecular mechanisms of selenium-mediated down-regulation of AR signaling are not clear. AR is a ligand-dependent transcription factor. The activation of AR requires binding to its ligand, translocation to the nucleus, and interaction with coregulators, including coactivators and corepressors, in the *AR* target genes. In this study, a systemic approach was taken to examine the modification of androgen signaling by selenium in human prostate cancer cells. The results suggest that selenium affects AR signaling at multiple levels, including AR mRNA expression, mRNA stability, protein degradation, nuclear translocation, and recruitment of coregulators.

Materials and Methods

Selenium Reagent and Cell Culture

Methylseleninic acid was synthesized as described previously (11). Human LNCaP prostate cancer cells were obtained from the American Type Culture Collection (Manassas, VA) and maintained in RPMI 1640 supplemented with 10% fetal bovine serum. The cells were grown at 37°C in 5% CO_2 and 95% air.

Cytosolic and Nuclear Protein Preparation

LNCaP cells were cultured in charcoal-stripped fetal bovine serum for 3 days. The cells were treated with 10 nmol/L DHT in the absence or presence of 10 μmol/L methylseleninic acid for 2 hours. Cells were harvested, washed with PBS twice, and resuspended in a hypotonic buffer [10 mmol/L HEPES-KOH (pH 7.9), 1.5 mmol/L $MgCl_2$, 10 mmol/L KCl, and 0.1% NP40] and incubated on ice for 10 minutes. Nuclei were precipitated by $3,000 \times g$ centrifugation at 4°C for 10 minutes. The supernatant was collected as the cytosolic fraction. After washing once with the hypotonic buffer, the nuclei were lysed in a lysis buffer [50 mmol/L Tris-HCl (pH 8), 150 mmol/L NaCl, 1% Triton X-100] and incubated on ice for 30 minutes. The nuclear

lysate was precleared by 10,000 rpm centrifugation at 4°C for 15 minutes. Protein concentration was determined using the Coomassie Plus protein assay kit (Pierce, Rockford, IL).

Northern Blot Analysis

Total RNA was extracted from cells with TRIzol reagent (Life Technologies, Rockville, MD). Twenty micrograms of each sample were electrophoresed on 1.2% denaturing agarose gels and transferred to a nylon membrane (MSI, Westborough, MA). A 500-bp fragment of AR cDNA was labeled with [α-^{32}P]dCTP (3,000 Ci/mmol; ICN, Costa Mesa, CA) using the Ready-To-Go DNA Labeling Beads (Amersham Pharmacia Biotech, Piscataway, NJ). Hybridization was carried out during 3 hours at 65°C in Rapid-hyb buffer (Amersham, Arlington Heights, IL). Membranes were washed for 15 minutes at 65°C in 2× SSC, 0.1% SDS (twice), 0.5× SSC, 0.1% SDS and 0.1× SSC, and 0.1% SDS. Radioactivity in the membranes was analyzed with a Storm Phosphoimager System.

Western Blot Analysis

The protein extracts were resolved on 12.5% SDS-PAGE. Proteins were then transferred to nitrocellulose membrane. After blocking overnight at 4°C in 5% milk in PBS/0.1% Tween 20, membranes were incubated for 1 hour at room temperature with anti-AR rabbit polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA) or anti-α-actin (Sigma, St. Louis, MO), anti-RNA polymerase II (Promega, Madison, WI), or anti-Hsp90 (Sigma) diluted in 1% milk in PBS/Tween 20. Following secondary antibody incubation, immunoreactive proteins were visualized with an enhanced chemiluminescence detection system (Amersham Pharmacia Biotech, Buckinghamshire, England).

In vitro AR Ligand-Binding Assay

Ligand-binding assay was done as previously described (19). LNCaP prostate cancer cells were plated at 1×10^6 per plate in 10-cm plates and allowed to grow to confluence for 3 days. Cells were treated with 10 μmol/L methylseleninic acid for 4 hours before harvesting and homogenization in TEDG buffer [10 mmol/L Tris (pH 7.4), 1.5 mmol/L EDTA, 10% glycerol, and 1 mmol/L DTT added immediately before use]. The cell suspension was passed through a 26-gauge needle (10–15 times) to homogenize. The homogenate was incubated on ice for 10 minutes and centrifuged at 13,000 rpm for 30 minutes at 4°C. The supernatant was collected and used as the cytosolic fraction. Total protein was estimated in the extracts from both untreated and methylseleninic acid–treated cells, and equal amounts of protein were used in the subsequent assay. The extracts were incubated with 0.01, 0.05, 0.1, 0.25, 0.5, 0.75, and 1 nmol/L ^{3}H-R1881 either in the presence or absence of 100 nmol/L (excess) unlabeled (cold) R1881 in a total reaction volume of 250 μL (made up with TEDG buffer). The reaction mixtures were incubated on ice throughout the assay. Dextran-coated charcoal suspension (500 μL; 0.25% charcoal, 0.025% dextran in 1× PBS) was added to each sample and incubated at 4°C with vigorous shaking for 10 minutes. The samples were centrifuged at 3,000 rpm

for 10 minutes at 4°C. The supernatant (500 μL) was added to 5 mL of scintillation fluid and counted in a liquid scintillation counter. The amount of the radio-labeled ligand bound to the receptor in the presence and absence of competing unlabeled ligand was calculated and expressed as fmol/mg protein. The difference between count per minute with ^{3}H-R1881 only and count per minute with ^{3}H-R1881 + cold R1881 was calculated and taken as the amount of bound ^{3}H-R1881. The data were analyzed by Scatchard analysis as described previously (19).

AR mRNA Stability Assay

Equal numbers of LNCaP cells were plated in 10-cm plates and incubated at 37°C until they reached 70% confluence. Cells were either pretreated with 5 μg/mL actinomycin D before treatment with 10 μmol/L methyl-seleninic acid, or they were treated with 5 μg/mL antinomycin D and 10 μmol/L methylseleninic acid together for 0, 4, 8, 12, 24, 36, and 48 hours. Total RNA was extracted with TRIzol reagent (Invitrogen, San Diego, CA), and 20 μg of total RNA from each sample were run on a 1.2% formaldehyde-agarose gel. The membrane was hybridized with the AR cDNA probe labeled with ^{32}P-dCTP. After hybridization and washing, radioactivity in the membranes was analyzed with a Storm Phosphorim-ager System, and the levels of AR mRNA were quantified by Phosphorimager. The turnover of AR mRNA was determined by comparing mRNA levels over time in cells treated with or without methylseleninic acid.

AR Protein Stability Assay

Equal numbers of LNCaP cells were plated in 60-mm plates and incubated at 37°C until they reached 70% confluence. Cells were either pretreated with 50 μg/mL cycloheximide before treatment with 10 μmol/L methyl-seleninic acid, or they were treated with 50 μg/mL cycloheximide and 10 μmol/L methylseleninic acid simul-taneously for 0, 4, 8, 12, 24, 36, and 48 hours. Cells were homogenized in high salt buffer [10 mmol/L HEPES (pH 7.5), 0.4 mol/L NaCl, 1 mmol/L EDTA, 1% NP40], and the supernatants were used as the whole-cell lysates. Equal amounts of protein were run on 10% SDS-PAGE and probed with anti-AR rabbit polyclonal antibody (Santa Cruz Biotechnology) or anti-α-actin antibody (Sigma). To determine if the proteasomal degradation pathway played a role in the degradation of AR protein in cells treated with methylseleninic acid, cells were treated with 5 μmol/L MG-132 (a proteasome inhibitor) in addition to cycloheximide. The levels of AR protein were quantified and normalized to the amount of actin. The AR protein turnover was determined by comparing AR protein levels over time in cells treated with or without methylseleninic acid.

Chromatin Immunoprecipitation Assay

LNCaP cells were cultured in phenol red-free RPMI 1640 supplemented with 10% charcoal-stripped fetal bovine serum for 3 days. Cells were treated with or without 10 μmol/L methylseleninic acid for 4 hours in the absence or presence of 10 nmol/L DHT. The AR and coregulator complexes were cross-linked inside the cells by the addition of formaldehyde (1% final concentration) to the cells in culture. Whole-cell extracts were prepared using sonication and an aliquot of the cross-linked receptor protein complexes were immunoprecipitated by incubation with either the AR specific antibody (AR441, Santa Cruz Biotechnology) or antibody that specifically recognize coactivator TIF-2, SRC-1, or corepressors, such as SMRT or NCoR (Santa Cruz Biotechnology), overnight at 4°C with rotation. Chromatin-antibody complexes were isolat-ed from solution by incubation with protein G-Sepharose beads for 1 hour at 4°C with rotation. The Sepharose-bound immune complexes were washed and eluted from beads with elution buffer (1% SDS and 0.1 mol/L NaHCO$_3$), and DNA was extracted. DNA samples from chromatin immunoprecipitation preparations were ana-lyzed by PCR using primers spanning the *PSA* gene in the region of promoter (forward, 5′-CCTAGATGAA-GTCTCCATGAGCTACA; reverse, 5′-GGGAGGGAGAGC-TAGCACTTG).

Results

Methylseleninic Acid Decreases AR mRNA Stability

Our results suggest that whereas methylseleninic acid decreased AR mRNA levels at the transcriptional level (14), AR mRNA expression can also be regulated at post-transcriptional level. To examine whether methylseleninic acid affects AR mRNA stability, LNCaP cells that express functional AR were treated with or without 5 μmol/L methylseleninic acid in the presence of actinomycin D (5 μg/mL) to stop *de novo* mRNA synthesis. The total RNA was isolated at different time points, and AR mRNA levels were measured by Northern blot analysis. The half-life of AR mRNA was determined by comparison of mRNA levels over time between cells treated with or without actinomycin D, either in the presence or absence of methylseleninic acid. Because actinomycin D is capable of inducing cell death, we monitored cell growth for a period of 24 hours and did not observe cell death or growth inhibition with the concentration of actinomycin D used (5 μg/mL). We did not observe significant cell death or growth inhibition at 5 μmol/L methylseleninic acid over a period of 24 hours in LNCaP cells (14). Methyl-seleninic acid treatment initially enhanced AR mRNA levels within 6 hours. However, AR mRNA levels were significantly decreased by methylseleninic acid compared with the control at 8 hours. Figure 1 shows on the semilog plot, the mean values of percentage of AR mRNA levels over time relative to respective time 0 AR mRNA value as 100%. In methylseleninic acid–treated cells, AR half-life was reduced to about 7 hours from 12 hours in the control cells, suggesting that AR mRNA degradation was greatly accelerated in the presence of methylseleninic acid after 6 hours.

Methylseleninic Acid Increases AR Protein Turnover

We have shown that methylseleninic acid decreased the levels of AR mRNA and protein in LNCaP cells (14). We
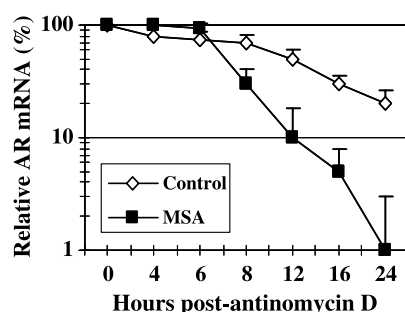
Figure 1. Effect of MSA on AR mRNA stability in LNCaP cells. The mRNA synthesis inhibitor antinomycin D (5 μg/mL) was added with or without 5 μmol/L methylseleninic acid (*MSA*) at time 0. At specific time points, cells were harvested, and total RNA was isolated by Northern blots. *Points*, means of three independent experiments plotted on semilog scale relative to respective time 0 AR mRNA value as 100%; *bar*, SD.

next examined the effect of methylseleninic acid on AR protein degradation after new protein synthesis was blocked by cycloheximide as a potential mechanism for down-regulation of AR protein level. The protein synthesis inhibitor cycloheximide (50 μg/mL) was added with or without 5 μmol/L methylseleninic acid at time 0. At specified time points, cells were harvested, and the levels of AR protein were measured by Western blot using anti-AR antibody. In methylseleninic acid–treated cells, the half-life of AR protein was reduced to 6 hours from 21 hours in the control cells (Fig. 2A), suggesting that AR protein degradation was greatly enhanced in the presence of methylseleninic acid. Systematic protein degradation by the ubiquitin-proteasome system plays an important role in the maintenance of protein stability. Protein ubiquitination provides the recognition signal for the 26S proteasome, leading to protein degradation (20, 21). Studies showed that AR protein level in cells is regulated by systemic protein degradation pathways (22, 23). To examine whether selenium induced AR protein degradation via ubiquitin-proteasome system, the 26S proteasome inhibitor MG132 was added to the cells treated with methylseleninic acid. MG132 was able to retard methylseleninic acid effect on AR protein levels (Fig. 2B), suggesting that methylseleninic acid induced AR degradation via a proteasome-dependent pathway.

Selenium Inhibits AR Nuclear Translocation

AR typically translocates to the nucleus to exert its function on gene expression. To examine whether selenium affects the translocation of AR, Western blot analysis was done using cell extracts from either cytosolic or nuclear extracts. LNCaP cells were cultured in charcoal-stripped fetal bovine serum for 3 days before adding 10 nmol/L of DHT in the absence or presence of 10 μmol/L methyl-seleninic acid for 2 hours. Nuclear and cytosolic fractions were prepared and used for Western blot analysis using the anti-AR antibody. DHT treatment increased the levels of AR protein expression in the nucleus, which were reduced by the treatment with methylseleninic acid (Fig. 3). In contrast, methylseleninic acid had little effect on AR

protein expression in the cytosol. The expression of RNA polymerase II and Hsp90 were used as markers for the integrity of the nuclear and cytosolic fractions, respectively. These results suggest that methylseleninic acid suppresses AR signaling in part via interruption of AR nuclear translocation.

Selenium Inhibits the Recruitment of Coactivators and Enhances the Recruitment of Corepressors to AR Target Genes

AR interacts with coregulators to achieve maximal transactivation activity. To examine the effects of selenium on the recruitment of coregulators to the promoters of AR target genes, chromatin immunoprecipitation analysis was done. DHT increased the recruitment of AR and TIF-2 and SRC-1 to the promoter of the *PSA* gene in the absence of methylseleninic acid, and this recruitment was greatly diminished in the presence of 5 μmol/L methylseleninic acid (Fig. 4). On the other hand, methylseleninic acid treatment prevented the nuclear translocation of AR in the presence of hormone; thus, the corepressors, including SMRT and NcoR, remain bound to the promoter of the *PSA* gene (Fig. 4). These results suggest that methylsele-ninic acid–mediated reduction of AR activation may be due, at least in part, to a decrease in the recruitment of AR and its coactivators to the promoter of the AR target gene *PSA*, while maintaining corepressors bound to the promoter.
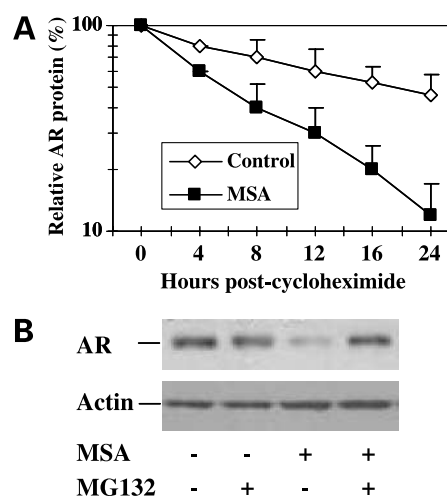

Figure 2. **A,** effect of methylseleninic acid (*MSA*) on AR protein turnover in LNCaP cells. The protein synthesis inhibitor cycloheximide (50 μg/mL) was added with or without 5 μmol/L methylseleninic acid at time 0. At specific time points, cells were harvested, and cell lysates were prepared. AR protein levels were determined by Western blot analysis using antibody specifically against AR and normalized to α-actin control. *Points*, means of three independent experiments plotted on semilog scale relative to respective time 0 AR value as 100%; *bars*, SD. **B,** effect of MG132 on methylseleninic acid–induced AR protein degradation. MG132 (5 μmol/L) was added to LNCaP cells together with cycloheximide (50 μg/mL) in the presence and absence of 5 μmol/L methylseleninic acid. The cell lysates were prepared at 24 h. AR protein levels were determined by Western blot analysis using antibodies specifically against AR and α-actin as a control.
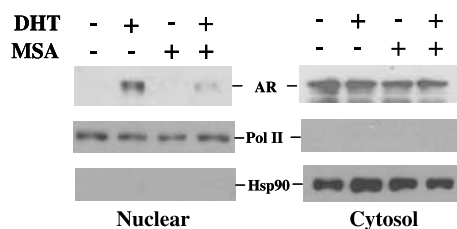
Figure 3. The effect of methylseleninic acid (*MSA*) on AR nuclear translocation. LNCaP cells were cultured in charcoal-stripped FBS for 3 d and treated with 10 nmol/L DHT with or without 10 μmol/L methylseleninic acid for 2 h. The cells were harvested for preparation of cytosolic and nuclear fractions and analyzed by Western blotting using antibodies against AR, polymerase II, or Hsp90. The expression of polymerase II and Hsp90 were used as markers for the integrity of the nuclear and cytosolic fractions, respectively.

Discussion

Selenium is an important trace element exhibiting anticancer activity. There are a number of potential mechanisms proposed for the anticancer effects of selenium, including antioxidant effects, enhancement of immune function, stimulation of apoptosis, and induction of cell cycle arrest (16). We previously showed a novel mechanism of selenium action in which selenium disrupts androgen signaling by inhibiting AR mRNA and protein expression and reducing the expression of AR target genes (14). These studies provide an important molecular mechanism of selenium chemoprevention and potential therapy for prostate cancer. In the present study, the mechanisms of selenium-mediated AR signaling down-regulation were examined. Selenium decreased AR mRNA stability, accelerated AR protein degradation, and blocked AR nuclear translocation. In addition, selenium inhibited the recruitment of coactivators and maintained corepressors bound to the promoters of AR target genes.

AR is a ligand-dependent transcription factor whose activation is initiated by its binding to androgen and subsequent translocation to the nucleus, where it binds to the promoters and activates the transcription of AR target genes. Any interruption of this process may alter AR signaling and result in abnormal androgen action. To examine whether selenium affects AR ligand binding, *in vitro* AR binding activity was done using ^{3}H-labeled R1881 and was subjected to Scatchard analysis in the absence and presence of 5 μmol/L methylseleninic acid in LNCaP cells. The results showed that selenium did not affect R1881 binding to AR (Fig. 5). Because LNCaP cells express a mutant AR, LAPC-4 cells containing a wild-type AR were used for AR ligand binding assay and selenium did not affect R1881 binding to AR in LAPC-4 cells (data not shown). The fact that selenium does not affect AR ligand binding suggests a different antiandrogen mechanism by selenium from flutamide or Casodex, which block ligand binding to AR (24).

AR transactivation may require cooperation with many other coregulators including coactivators and corepressors. It is known that androgen-AR may cooperate with various
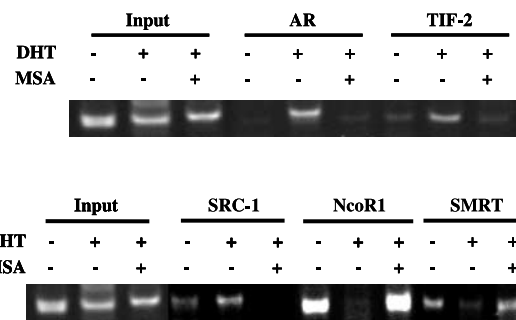

Figure 4. Effect of methylseleninic acid (MSA) on the recruitment of AR and coregulators to the promoter of an endogenous AR target gene *PSA*. The *in vivo* binding of AR and coregulators to the PSA promoter was examined by the chromatin immunoprecipitation assay. LNCaP cells were cultured in charcoal-stripped condition for 3 d. Soluble chromatin was prepared from cells treated with 10 nmol/L DHT for 4 h (+) or untreated (−) in the presence (+) or absence (−) of 10 μmol/L methylseleninic acid and immunoprecipitated with antibodies against AR, TIF-2, SRC-1, SMRT, and NCoRI. Coprecipitated DNA was amplified by PCR using primers that flank the ARE in the PSA promoter region. The presence of total PSA promoter DNA in the soluble chromatin before immunoprecipitation was included as input.

coregulators to modulate their target genes for proper or maximal function. Coregulators such as TIF-2 and SRC-1 interact with AR to enhance ligand-dependent transactivation of AR. The expression of TIF-2 and SRC-1 is increased in cancer and recurrent prostate cancer after medical or surgical castration (25), suggesting that TIF2 and SRC-1 may be involved in the development and progression of prostate cancer. Our findings showed that selenium can interrupt the interaction between AR and coregulators by blocking the recruitment of coactivators (SRC-1 and TIF-2) while maintaining corepressors (SMRT and NCoR) bound to the promoters of AR target genes. These findings suggest that selenium not only disrupts AR signaling, but also interrupts the interaction of coregulators with AR to achieve maximal effect on androgen function.

A common treatment modality for prostate cancer is androgen deprivation, which can be achieved by surgical
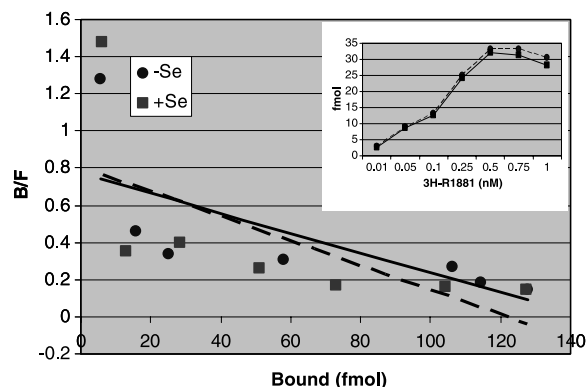

Figure 5. Scatchard analysis of specific R1881 binding to AR in LNCaP cells in the absence and presence of 5 μmol/L methylseleninic acid (*Se*). *Points,* means from triplicate experiments; *bars,* SE. *Inset,* saturation binding results.

castration, chemical castration, or a combination of surgical and chemical castrations. The goal of these androgen deprivation treatments is either to block androgen-AR binding or to reduce the levels of androgen. Although antiandrogen treatment is effective, the antitumor effects may be temporary. Virtually, every patient will relapse due to the growth of androgen-independent prostate cancer cells. There is an urgent need for testing new therapies based on different mechanisms to target AR signaling for androgen-independent prostate cancer. AR signaling is often hyperactive in androgen-independent prostate cancer and plays a critical role in the growth and progression of prostate cancer. A treatment aims at reducing AR expression may represent an attractive approach to target androgen signaling in prostate cancer. Our findings show that selenium disrupts androgen signaling at multiple stages of AR signaling pathways, including AR mRNA expression, mRNA stability, protein degradation, nuclear translocation, and interaction with coregulators in prostate cancer (14). This unique antiandrogen activity suggests that selenium may serve as a therapeutic agent, in addition to a chemopreventive agent, for prostate cancer. Understanding the molecular mechanism of selenium-mediated down-regulation of AR signaling may aid in the development of effective treatments aimed at targeting AR signaling for prostate cancer. We are currently testing the combination treatment to more effectively target AR signaling in prostate cancer using antiandrogen agents (flutamide or Casodex, blocking ligand binding to AR) and selenium (reducing AR expression) based on our understanding of the mechanisms of their action.

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Abstract

The growth of prostate epithelial cells requires physiological levels of androgen, both to stimulate proliferation and inhibit apoptotic death. Androgen binds to the androgen receptor (AR), which causes AR to bind to androgen responsive elements (AREs) in the promoters of androgen-regulated genes. This interaction is affected by many other transcription coregulators. These complex interactions between AR, AREs and coregulators facilitate the activation or repression of genes regulating development, differentiation, and proliferation of target cells. Several androgen-responsive genes have been identified, including prostate specific antigen (PSA) and human glandular kallikrein 2 (hk2).

Selenium is an essential nutrient that has a chemopreventive effect against a variety of malignancies including prostate cancer. A number of case-controlled epidemiological studies have demonstrated an inverse relationship between selenium status and prostate cancer risk. One of the most important studies of selenium as a chemopreventive agent is the Nutritional Prevention of Cancer (NPC) study initiated by Larry Clark. Supplementation of people with selenized yeast was capable of reducing the overall cancer morbidity by nearly 50%. Although selenium treatment did not significantly affect the incidence of non-melanoma skin cancers, patients receiving the supplement showed a significantly lower prevalence of developing lung (RR=0.54), colon (RR=0.42) or prostate cancer (RR=0.37). Further analysis re-affirmed the significant reduction in prostate cancer incidence by selenium (RR=0.48, 95% CI = 0.28-0.80). The promising epidemiological and prevention studies on selenium in prostate cancer provide the basis for the current Selenium and Vitamin E Chemoprevention Trial (SELECT).

The biological activity of selenium is dependent on its chemical form. In general, inorganic selenium compounds, such as selenate or selenite, are known to produce genotoxic effects. Organic selenium-containing compounds such as selenomethionine, methylselenocysteine (MSC), are better tolerated and exhibit anti-carcinogenic activity. Methylseleninic acid (CH3SeO2H, abbreviated as MSA) was developed specifically for *in vitro* studies, since cultured cells respond poorly to selenomethionine (a commonly used selenium reagent) due to very low levels of β-lyase activity which is required for conversion of selenomethionine to the active methylselenol. The effect of physiological concentrations of MSA on cultured cells has been documented in several studies.

Cell culture studies demonstrated that selenium inhibited the growth of prostate cancer cell lines including androgen sensitive LNCaP and androgen insensitive DU145 and PC3 cells. *In vivo* studies also support the antitumorigenic role of selenium in prostate cancer. Dietary supplementation of selenium resulted in reduction of tumor growth in PC3 tumors in mice. There are a number of potential mechanisms proposed for the anti-proliferative effects of selenium, including antioxidant effects, enhancement

of immune function, stimulation of apoptosis, and induction of cell cycle arrest. We recently demonstrated that MSA is able to decrease markedly AR transcript and protein levels. The expression of prostate-specific antigen (PSA), a well-known androgen-regulated gene, is also inhibited by MSA.

The down regulation of AR signaling by selenium provides an important mechanism for selenium prostate cancer chemoprevention. However, the molecular mechanisms of selenium-mediated down regulation of AR signaling are not clear. AR is a ligand-dependent transcription factor. The activation of AR requires binding to its ligand, translocation to the nucleus, interaction with coregulators including coactivators and corepressors in the AR target genes. In this study, a systemic approach was taken to examine the modification of androgen signaling by selenium in human prostate cancer cells. In addition, the effect of selenium (methylselenocysteine) on human prostate cancer cell tumor growth was examined. The results suggest that selenium affects AR signaling at multiple levels including AR mRNA expression, mRNA stability, protein degradation, nuclear translocation, and recruitment of coregulators. Pharmacological dose of methylselenocysteine inhibits the growth of LNCaP human prostate cancer *in vivo* accompanied by a decrease in the expression of androgen receptor and PSA.